Note

Pheophorbide-a acts as a photodynamic DNA-damaging reagent that causes remarkable increase in 8-hydroxydeoxyguanosine in cellular DNA

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要旨：クロロフィル a の誘導体であるフェオホルバイド a は、クロロフィル分解物を含む食品による食中毒に伴う光線過敏症を引き起こすが、癌の光線力学的療法のための強力な光増感剤としても期待されている。本研究では、可視光照射下においてフェオホルバイド a 処理したヒト胃癌由来の KATO III 細胞から抽出した DNA 中に、主要な酸化的 DNA 損傷の 1 つである 8-ヒドロキシデオキシグアノシン（8OH-dG）が顕著に増加することを見出した。好気性条件下でフェオホルバイド a の存在下で細胞を可視光で照射すると、8OH-dG が時間依存的に感作細胞の DNA 中で増加した。また、フェオホルバイド a、光、または酸素のいずれかが欠乏していると、8OH-dG の生成は顕著に抑制されたことから、8OH-dG の生成がフェオホルバイド a 介在性の光線力学的酸化に依ることが示された。これらの知見は、一重項酸素などの活性酸素種がフェオホルバイド a 存在下での光線力学的反応によって発生し、細胞内での 8OH-dG の生成を促進することを示唆している。

キーワード：フェオフォルバイド a、光線力学的療法、8-ヒドロキシデオキシグアノシン、光酸化、DNA 損傷

Summary

Although pheophorbide-a derived from chlorophyll a brings about photosensitivity disorders such as food poisoning due to the intake of food containing chlorophyll degradation products, it has also been considered as a strong photosensitizer for the photodynamic therapy of cancers. Data obtained in this study revealed that the amounts of 8-hydroxydeoxyguanosine (8OH-dG), one of the major oxidative DNA damage, were increased in DNA extracted from pheophorbide-a-treated cultured human stomach cancer KATO III cells under visible light irradiation. 8OH-dG levels were time-dependently increased in DNA of sensitized cells when the cells were irradiated with visible light in the presence of pheophorbide-a under aerobic condition. Lack in either one of the dye, light or oxygen resulted in little formation of 8OH-dG, indicating that formation of 8OH-dG is dependent on pheophorbide-a-mediated photodynamic oxidation. These findings suggested that reactive oxygen species such as singlet oxygen are generated by photodynamic reaction in the presence of pheophorbide-a, resulting in accelerated formation of 8OH-dG in cells.

Key words: pheophorbide-a, photodynamic therapy, 8-hydroxydeoxyguanosine, photo-oxidation, DNA damage

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Introduction

8-Hydroxydeoxyguanosine (8OH-dG) is one of the major single base damage in DNA[1]. 8OH-dG is well known to be formed by various reactive oxygen species (ROS) producing reagents, such as reducing agents, X-rays, asbestos plus hydrogen peroxide, and so on[2], resulting in point mutations in DNA[3]. On the other hand, visible light-irradiation also can promote formation of 8OH-dG in DNA in the presence of various organic compounds such as methylene blue[4], thiazin[5], phthalocyanine[6] and riboflavin[7].

Pheophorbide-a is a degradation product of chlorophyll a, and lacking in a magnesium ion and a phytol, and its maximum absorption wavelength is 666 nm[8]. By visible light-irradiation, ROS such as singlet oxygen are generated in the presence of pheophorbide-a[9]. Pheophorbide-a has been known not only as a poison which causes photosensitivity disorders[10] but also as a hopeful photosensitizer for photodynamic therapy of cancers[11]. During pre-incubation of cancer cells with pheophorbide-a, it accumulates in cytoplasm of the cells[12]. Following light-irradiation brings about generation of ROS and release of cytochrome *c* from mitochondria, resulting in apoptotic cell death[13),14]. However, the effect of pheophorbide-a-mediated photodynamic reaction on 8OH-dG generation remains to be solved.

In this paper, I found great increase in content of 8OH-dG in DNA of photosensitized cells when the cells were illuminated with visible light in the presence of pheophorbide-a under aerobic condition.

Materials and Methods

1. Cell and reagents

KATO III cells established from a lesion of a patient with gastric signet ring carcinoma[15] were supplied from Japan Cell Resource Bank. Cells were cultured in RPMI-1640 medium containing 10% fetal calf serum. Pheophorbide-a was supplied by Yakult (Tokyo, Japan). Nuclease P1 and *Escherichia coli* alkaline phosphatase were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Photo-oxidation of KATO III cells

Cells were harvested by centrifugation, washed twice with phosphate-buffered saline, suspended in HEPES-saline buffer, and preincubated with 25 μM pheophorbide-a for 1 h. Constantly-stirred cells in a tube (4 x 10^6 cells in 0.35 mL buffer) were illuminated with a 150 W fiber-optic halogen lamp (Nikon, Tokyo, Japan) from a distance of 3 cm. In control experiments, pheophorbide-a was omitted. Regarding removal of dissolved oxygen from the cell suspension, air was replaced by nitrogen (N_2) gas by constant stirring of the suspension under the flow of 100% N_2 gas for 10 min.

3. DNA extraction and quantitative analysis of 8OH-dG

Cells collected by centrifugation were injected into a nucleic acid extractor Model 340A (Applied Biosystems, Foster City, CA, USA) for the extraction of DNA. DNA samples were dissolved in 100 μL of 20 mM sodium acetate buffer (pH 4.8), digested by 4 μg of nuclease P1 at 37˚C for 1 h, and treated with 0.2 unit of *E. coli* alkaline phosphatase in 100 mM Tris-HCl buffer (pH 7.15) at 37˚C for 1 h. Resulting deoxynucleoside mixtures were applied to a high performance liquid chromatography system HLC-803D (Tosoh, Tokyo, Japan) equipped with an octadecylsilyl-silica gel column (0.46 x 25 cm; Beckman Coulter Inc., Brea, CA, USA). Deoxynucleosides were eluted by 8% (v/v) aqueous methanol containing 10 mM Na_2HPO_4 at a flow rate of 1 mL/min. Deoxyguanosine and 8OH-dG were simultaneously monitored by a UV detector UV-8 (Tosoh) at 290 nm and an electrochemical detector EC-8000 (Tosoh), respectively. The molar ratio of 8OH-dG to deoxyguanosine in each DNA sample was determined as described[2].

4. Statistical analysis

Data are presented as averages of three separate

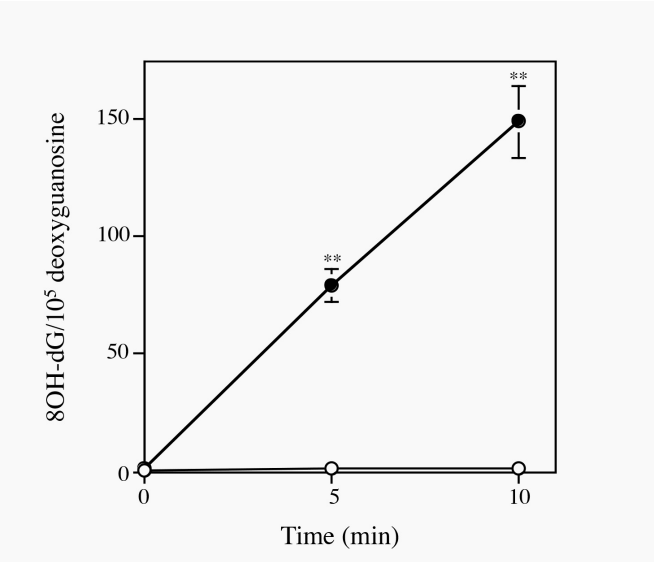

Fig. 1. Formation of 8OH-dG by pheophorbide-a-mediated photo-oxidation in cellular DNA in a time-dependent manner. KATO III cells were incubated with (closed circles) or without (open circles) 25 μM pheophorbide-a, and photo-irradiated up to 10 minutes. The determination of the content of 8OH-dG in DNA and statistical data analysis were performed as described in "Materials and Methods." Statistical differences between "with pheophorbide-a" and "without pheophorbide-a" at each time point were calculated with Student's *t* test. ** $P < 0.01$.

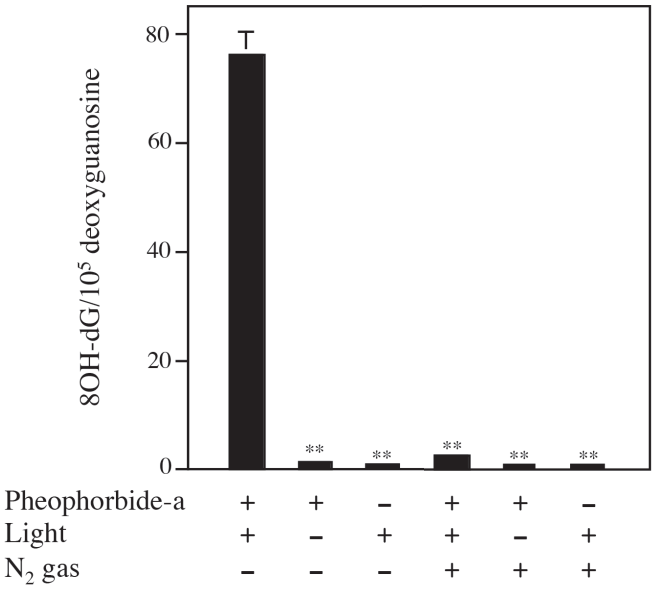

Fig. 2. Requirement of pheophorbide-a, light-irradiation and oxygen for generation of 8OH-dG. KATO III cells were incubated under the conditions lacking in either one of the pheophorbide-a, light or oxygen for 5 min. The determination of the content of 8OH-dG in DNA and statistical data analysis were performed as described in "Materials and Methods." ** $P < 0.01$, compared with the data in the presence of pheophorbide-a, light and oxygen (the leftmost bar).

experiments. Error bars indicate standard deviation. Statistical differences were calculated with Student's *t* test.

Results and Discussion

KATO III cells were photo-irradiated in the presence of pheophorbide-a. Figure 1 shows that photosensitized formation of 8OH-dG depends on the presence of pheophorbide-a. In the presence of pheophorbide-a, the amounts of 8OH-dG were linearly increased in time-dependent manner up to 10 min, and reached to about 0.15% of the total deoxyguanosine in cellular DNA. In contrast, no changes in the amounts of 8OH-dG were observed in the absence of pheophorbide-a.

Requirement of oxygen for the photo-oxidation mediated by pheophorbide-a was examined by substitution of dissolved oxygen to N_2. Figure 2 shows that not only lack in either pheophorbide-a or light-irradiation but also removal of oxygen resulted in little formation of 8OH-dG. These results revealed that both light and oxygen are essential for the formation of 8OH-dG in KATO III cells in the presence of pheophorbide-a, indicating this oxidative reaction completely dependent on photodynamic process.

In this paper, I demonstrated that 8OH-dG is remarkably generated in nuclear DNA of photosensitized KATO III cells when the cells are illuminated with visible light in the presence of pheophorbide-a under aerobic condition. Previous study showed that pheophorbide-a distributes only in cytoplasm, but not in nuclei[12]. However, interestingly, the results obtained in this study suggested that pheophorbide-a-mediated photo-oxidation can promote damage to not only mitochondria[13),14)] but also DNA in nuclei. Perhaps, some kind of intracellular factor may be involved in the effective pheophorbide-a-mediated photodynamic oxidation of genomic DNA resulting in accelerated formation of 8OH-dG in nuclei. For example, phospholipids may be the strong candidates that enhance formation of 8OH-dG by pheophorbide-a-mediated photodynamic oxidation. However, participation of phospholipids in this oxidation of DNA remains poorly understood. The mechanism of enhancement of 8OH-dG generation in nuclear DNA should be elucidated in the future.

The pheophorbide-a-mediated photo-oxidation can be expected to show strong cytotoxicity against various solid cancers that take up pheophorbde-a. The results obtained in this study may be significantly useful for elucidating molecular mechanisms of the cytotoxicity of pheophorbide-a, resulting in development of photodynamic therapy of cancers using pheophorbide-a.

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